Analysis of the DNA "mismatch-repair" enzyme human Mut-S-Homologon-2 in endometrial cancer on protein- and RNA-level

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Summary

Background: Microsatellite instability seems to be important in the development of various human cancers including sporadic endometrial cancer and is characterized by length changes at repetitive loci scattered throughout the genome. It has been shown that cancer predisposition is attributable to defects in any one of four genes, all of which encode homologs of the microbial mismatch repair proteins mutS and mutL.The human Mut-S-Homologon-2 gene (*h*MSH-2) specifies a mutS homolog, whereas hMLH-1, hPMS-1 and hPMS-2 encode homologs of mutL.

Material and Methods: Freshly excised endometrial specimens (malignancies of the uterine corpus: n=50; normal endometrial tissue: n=20) were examined by immunohistochemistry (mAb FE 11, Dianova, Germany) and RT-PCR to analyze the expression of human MUT-S-Homologon-2 on protein- and mRNA-level. Most of the neoplasms of the uterine corpus were sporadic endometrial.

Results: In the immunohistochemical study, 25% of normal endometrial tissues were human Mut-S-Homologon-2 negative, while the remaining 75% revealed weak human Mut-S-Homologon-2 immunoreactivity (mean human Mut-S-Homologon-2 IRS: 1.52; SD: ±1.42; mean human Mut-S-Homologon-2-PP: 12.12; SD: ±10.31; mean human Mut-S-Homologon-2 IS: 0.98; SD: ±0.81). All malignancies of the uterine corpus revealed strong nuclear immunoreactivity for human Mut-S-Homologon-2 (mean human Mut-S-Homologon-2-IRS: 9.12, SD: ±3.34; mean human Mut-S-Homologon-2-PP: 81.82, SD: ±15.67; mean human Mut-S-Homologon-2-IS: 2.58, SD: ±0.71). In addition, expression of human Mut-S-Homologon-2 protein was statistically significantly upregulated in tumor cells of malignancies of the uterine corpus as compared to normal endometrial tissue on the protein level. In the RT-PCR study, the *h*MSH-2 gene was highly expressed in endometrial neoplasms on the mRNA-level. *h*MSH-2 expression was consistently increased in endometrial neoplasms compared to normal endometrial tissue.

Conclusion: The expression of the human MUT-S-Homologon-2 is increased both on the protein- and on mRNA-level in endometrial neoplasms compared to normal endometrial tissue possibly caused by the neoplastic process driven by an increase in the rate of mutations in oncogenes and tumor suppressor genes.

Key words: DNA-mismatch repair; hMSH-2; Endometrial cancer.

Introduction

Instability of microsatellite DNA sequences was first described in a subset of sporadic colorectal cancers and in cancers associated with the familial cancer syndrome HNPCC (hereditary nonpolyposis colon cancer). Recently, it was demonstrated that microsatellite instability secondary to replication errors can be detected in various malignant tumours [1-8]. Endometrial cancer is the most common noncolorectal carcinoma occurring in women affected by HNPCC, and microsatellite instability has been observed in both the inherited form and in approximately 20% of presumably sporadic endometrial carcinomas [9-10].

Studies in microorganisms have shown that mutations in genes involved in DNA mismatch repair lead to instability of simple repetitive DNA sequences. These studies provided the initial link between mismatch repair abnormalities and the instability of microsatellite DNA sequences. In addition, when DNA-mismatch repair is lacking in microorganisms, the rate at which mutations accumulate is increased. The DNA mismatch repair system has been well characterized in microorganisms where it has been shown to detect and repair mispaired bases present in the cellular genome [5, 8].

Microsatellite instability seems to be important in the development of various human cancers and is characterized by length changes at repetitive loci scattered throughout the genome [5]. In most patients with hereditary nonpolyposis colon cancer (HNPCC), where almost every tumour reveals a high incidence of mutations in microsatellite repeat sequences, it was shown that cancer predisposition is attributable to defects in any one of four genes, all of which encode homologs of the microbial mismatch repair proteins mutS and mutL [6]. The human Mut-S-Homologon-2 gene specifies a mutS homolog, whereas hMLH-1, hPMS-1 and hPMS-2 encode homologs of mutL. Analysis of all 16 exons of human Mut-S-Homologon-2 in 34 unrelated HNPCC kindreds has revealed a heterogeneous spectrum of mutations [7]. Tumour cells that display microsatellite instability are typically defective in mismatch correction, thus providing a direct link between DNA-mismatch repair

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enzymes and genetic stability afforded by this DNA repair system [8].

Previously, we demonstrated that the expression of the DNA mismatch repair enzyme hMSH-2 seems to be increased on the protein level not only in endometrial neoplasms compared to normal endometrial tissue but also in other gynecological malignancies [11-16]. The aim of this study was to investigate the expression of human Mut-S-Homologon-2 in normal endometrial tissue and in endometrial neoplasms both on the protein level and on the RNA-level.

Materials and Methods

Endometrial specimens:

Freshly excised endometrial specimens (malignancies of the uterine corpus: n=50; normal endometrial tissue: n=20) were immediately embedded in OCT-Tissue-TEK II (Miles Scientific, Naperville, IL, USA), snap-frozen in liquid nitrogen, and stored at –80°C. Most of the neoplasms of the uterine corpus were sporadic endometrial adenocarcinomas, while five tumours were mixed epithelial-nonepithelial tumours (heterologous carcinosarcomas: n=5). All specimens of normal endometrial tissue were obtained from patients, who had to undergo surgery for leiomyomas of the uterine corpus. All of these patients were in the secretory menstrual phase. The histopathological diagnosis of normal endometrial tissue was confirmed by a specialized histopathologist.

Preparation of sections and fixation:

Serial sections (7 µm) were cut on a cryostat (Reichert-Jung, Heidelberg, Germany) and mounted on pretreated glass slides. Pretreatment of slides with 2% aminopropylmethoxysilane (Sigma, München, Germany) in acetone for 5 min was performed to enhance sticking of sections during the staining procedure. Sections to be strained for human Mut-S-Homologon-2 were fixed in 3.7% paraformaldehyde (Merck 4005; Darmstadt, Germany) in PBS (10 min at room temperature (RT), incubated in methanol (Merck 6009; 3 min, –20°C), and transferred into PBS.

Primary antibody:

Human Mut-S-Homologon-2 was detected by mouse monoclonal antibdoy FE11 (Dianova NA27, Hamburg, Germany).

In situ *detection of human Mut-S-Homologon-2 protein:*

Incubation steps were performed in a moist chamber at RT, covering the sections with 100 µl of the respective reagents. The slides were incubated with the human Mut-S-Homologon-2 antibody at a dilution of 1:20. After intermediate washing steps (PBS/TBS twice for 5 min), the sections were incubated with biotin-labeled rabbit anti-mouse IgG or goat anti rabbit IgG (Dako) at a diluation of 1:400 (30 min, RT) and incubated with streptavidin-peroxidase complexes (Dako, 1:400,30 min, RT). After rinsing, the sections were incubated with 3-amino-9-ethylcarbazole (AEC: Sigma A5754) as a substrate for the peroxidase reaction, transferred into tapwater, and mounted with Aquatex (Merck). In control sections, primary antibodies were replaced with polyclonal mouse IgG (Dako) or polyclonal rabbit IgG (Dako). No immunoreactivity was observed in the control sections. Specimens were analyzed under a Zeiss microscope (Zeiss, Oberkochen, Germany). Photographs were taken on Kodak Ektachrome 64 film.

Semi-quantitative analysis of immunoreactivity:

Microscopic analysis was performed by three independent observers including a specialized histopathologist. Human Mut-S-Homologon-2 staining intensity (human Mut-S-Homologon-2-SI), a percentage of human Mut-S-Homologon-2 positive cells (human Mut-S-Homologon-2-PP) and a resulting human Mut-S-Homologon-2 immunoreactivity score (human Mut-S-Homologon-2-IRS) were assessed as a modification of the technique described previously for estrogen and progesterone receptors [17]. In short, this immunoreactivity score (human Mut-S-Homologon-2-IRS: negative: 0-1; weak: 2-3; moderate: 4-6; strong: 8-12) was determined by multiplication of the values for human Mut-S-Homologon-2-staining intensity (human Mut-S-Homologon-2-SI: 0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining) and the values for percentage of human Mut-S-Homologon-2 positive tumour cells (human Mut-S-Homologon-2-PP: 1: 0% - 10%; 2: 11% - 50%; 3: 51% - 80%; 4: 81% - 100%.

Statistics:

Statistics were performed by using the Mann-Whitney U - Wilcoxon Rank Sum W Tests. Statistical significance was defined at $p<0.05$.

Reverse transcription polymerase chain reaction (RT-PCR) for hMSH-2 in normal endometrial tissue and in endometrial neoplasms:

Freshly excised endometrial specimens (malignancies of the uterine corpus: n=10; normal endometrial tissue: n=5) were immediately embedded in OCT-Tissue-TEK II (Miles Scientific, Naperville, IL, USA), snap-frozen in liquid nitrogen, and stored at –80°C. RNA was isolated using GITC as described previously [18].

Two micrograms each of total RNA from human endometrial neoplasms and normal human endometrial tissue were reverse transcribed according to the protocol for BRL's superscript preamplification system (GIBCO BRL, Gaithersburg, MD) for first strand cDNA synthesis. Ten percent of each cDNA reaction was used as a template in each sample. PCR sequence specific primer for hMSH-2 were used. Final reaction concentration was 0.2 µmol/l for each primer 0.2 µmol/l dNTPs, 1 x standard PCR buffer 2.5 µmol/l MgC12, and 0.75 units of AmpliTaq DNA polymerase in a final reaction volume of 30µl. Thermocycling conditions in a GeneAmp PCR System 9600 (Perkin Elmer) were as follows: two minutes initial denaturation at 94°C, followed by 30 cycles of denaturating at 95°C 20 seconds, annealing at 60°C 30 seconds, and extension at 72°C 30 seconds. A 10 minute incubation at 72°C after 30 cycles concluded the extension. PCR products were separated on 1.2% agarose gel.

Results

Expression of human Mut-S-Homologon-2 in normal endometrial tissue:

25% of normal endometrial tissues were human Mut-S-Homologon-2 negative, while the remaining 75% revealed weak human Mut-S-Homologon-2 immunoreactivity (mean human Mut-S-Homologon-2-IRS: 1.52; SD: ±1.42; mean human Mut-S-Homologon-2-PP: 12.12; SD: ±10.31; mean human Mut-S-Homologon-2-IS: 0.98; SD: ±0.81). In the endometrial specimens, the connective tissue always gave negative staining results. Fibroblasts, identified by their shape, revealed either negative or very

weak staining for human Mut-S-Homologon-2. Endothelial cells of capillaries, arterioles, and venules as well as pericytes or smooth muscle cells in the microvascular wall revealed either negative or very weak human Mut-S-Homologon-2-immunoreactivity as well [11, 12].

Expression of human Mut-S-Homologon-2 in malignancies of the uterine corpus:

All malignancies of the uterine corpus analyzed revealed strong nuclear immunoreactivity for human Mut-S-Homologon-2 (mean human Mut-S-Homologon-2-IRS: 9.12, SD: ±3.34; mean human Mut-S-Homologon-2-PP: 81.82, SD: ±15.67; mean human Mut-S-Homologon-2-IS: 2.58, SD: ±0.71). There was no visual difference in comparing the labelling pattern for human Mut-S-Homologon-2 in different types of malignancies of the uterine corpus. Tumour cells consistently revealed heterogeneous and strong human Mut-S-Homologon-2 immunoreactivity (Fig. 1). In addition, expression of human Mut-S-Homologon-2 protein was consistently and markedly upregulated in tumour cells of malignancies of the uterine corpus as compared to normal endometrial tissue (Fig. 1). This upregulation was statistically significant (human Mut-S-Homologon-2-PP: $p<0.001$, human Mut-S-Homologon-2-IS: $p<0.001$, human Mut-S-Homologon-2-IRS: $p<0.001$; Mann Whitney U Wilcoxon Rank Sum-Test).

RT-PCR Analysis of hMSH-2 in malignancies of the uterine corpus and in normal endometrial tissue:

The RT-PCR product of the *h*MSH-2 gene was a 410bp fragment; RT-PCR product of GAPDH was 720bp. We used an equal amount of total RNA for reverse transcription. The *h*MSH-2 gene was highly expressed in endometrial neoplasms. *h*MSH-2 expression was consistently increased in endometrial neoplasms compared to normal endometrial tissue. There was no visual difference in the GAPDH gene expression between normal human endometrial tissue and endometrial neoplasms (Fig. 2).

Discussion

Endometrial carcinoma is the most common type of gynecologic malignancy. It is thought to progress through a series of histologic changes from normal to hyperplasia and dysplasia before transformation [19]. Presumably there are corresponding underlying somatic mutations, although these alterations are poorly characterized and have been found in only a minority of tumours. Some of the most common mutations are found in the c-K-ras gene and the p53 gene [20-25].

Recent studies of colorectal cancers [26-29] have demonstrated a novel mechanism of tumourigenesis in which additions or deletions are ubiquitously present in simple base-pair repeat sequences or microsatellites. Instead of a few somatic mutations in critical oncogenes or tumour suppressor loci, there appear to be thousands of such microsatellite alterations distributed throughout the genome. This replication error is closely associated with hereditary nonpolyposis colorectal cancer [26, 28, 29] and has been linked to loci on chromosome 2p21 and 3p21-23 [30]. The locus on chromosome 2 has been identified as the hMSH-2 gene [6]. This gene is responsible for mismatch DNA repair after replication and cell lines derived from replication error positive tumours demonstrate increased microsatellite mutation rates [6, 31-34].

Endometrial cancers are the most common extracolonic manifestations observed in the families with HNPCC [35, 36]. Preliminary studies [10, 29] have demonstrated that most endometrial cancers arising in the setting of the Lynch syndrome and some sporadic endometrial cancers exhibit the replication error positive phenotype.

Previously, we reported for the first time on an extensive analysis of the expression of the DNA mismatch repair enzyme human Mut-S-Homologon-2 in malignancies of the uterine corpus using immunohistochemistry [11, 12]. Those results are strengthened through the examinations by immunohistochemistry and RT-PCR in this study. All endometrial carcinomas that were analyzed revealed strong nuclear immunoreactivity for human Mut-S-Homologon-2. Additionally, our results revealed upregulation of human Mut-S-Homologon-2 protein in malignancies of the uterine corpus as compared to normal endometrial tissue on the protein level detected by immunohistochemical examinations. As shown in this study, the expression of the DNA mismatch repair enzyme human Mut-S-Homologon-2 in malignancies of the uterine corpus is increased – compared to normal endo-

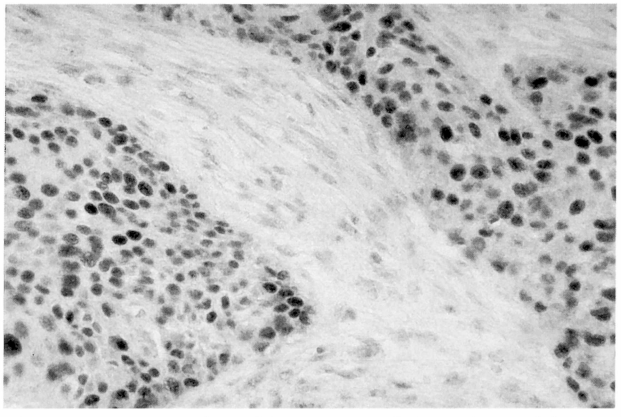

Figure 1. — Expression of human Mut-S-Homologon-2 in mucinous adenocarcinoma of the endometrium. Note the strong nuclear immunoreactivity for human Mut-S-Homologon-2. Magnification x400.

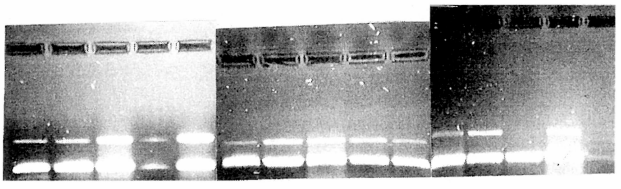

Figure 2. — Semiquantitative RT-PCR analysis of human Mut-S-Homologon-2 in endometrial adenocarcinoma (n=15; lane 6-15) and normal endometrial tissue (n=5; lanes 1-5). Note the consistently increased expression of human Mut-S-Homologon-2 mRNA in endometrial adenocarcinoma.

metrial tissue – not only on the protein level but also on the RNA-level using RT-PCR.

The results of this study, in which immunohistochemistry and RT-PCR were used to analyze the expression of the DNA mismatch repair enzyme hMSH-2 on the protein-and RNA-level, have therefore several important implications.

First, they suggest that all tumours that we have analyzed were not affected by inactivating mutations of the human Mut-S-Homologon-2 gene because the majority of inactivating mutations of human Mut-S-Homologon-2 lead to a lack of expression or the expression of a truncated protein *not* detectable by the antibody that we have used in this study [2, 5]. This fact adds to the body of evidence that mutations of the human Mut-S-Homologon-2 gene are not common in sporadic endometrial carcinomas [2, 37]. Preliminary studies suggested that microsatellite instability in sporadic endometrial carcinomas is not caused by mutations in the human Mut-S-Homologon-2 gene [2, 11, 12].

Second, our results suggest that the expression of functionally active human Mut-S-Homologon-2 is upregulated in sporadic endometrial carcinomas not only on the protein level as shown previously [11, 12] but also on the mRNA-level as demonstrated in this study. This human Mut-S-Homologon-2 upregulation on the protein- and RNA-level may be caused by the neoplastic process driven by an increase in the rate of mutations in oncogenes and tumour suppressor genes. Thus, it was recently shown that the human Mut-S-Homologon-2 gene is directly regulated by p53 [7]. The promotor region of the human Mut-S-Homologon-2 gene was sequenced and a p53 recognition site displaying strong wild type p53 binding activity was identified [7]. Transient cotransfection experiments using a human Mut-S-Homologon-2-promotor-reporter construct and a p53 expression plasmid confirmed the functional importance of this finding. These data demonstrated for the first time direct regulation of a DNA-mismatch repair gene by p53. Thus, upregulation of human Mut-S-Homologon-2 may be induced by an increase in p53 protein in malignancies of the uterine corpus cells.

Previously, we compared the expression of human Mut-S-Homologon-2 protein with that of Ki-67 antigen in sequential sections [11, 12]. In that study, tumour specimens revealed no visual correlation. These findings suggested, that expression of human Mut-S-Homologon-2 is not exclusively regulated by the proliferative activity of these tumour cells, but is influenced by different unknown mechanisms. It can be speculated, whether upregulation of human Mut-S-Homologon-2 in malignancies of the uterine corpus may be induced by other genes involved in post-replication recovery. Recently, it has been shown that mutations in distinct mutS and mutL genes can block nucleotide excision repair, indicating a direct contribution of DNA-mismatch repair enzymes to nucleotide excision repair pathways [38, 39].

Because nucleotide-excision repair removes damaged DNA (i.e. cross-linked DNA after many anticancer treatments), upregulation of biologically and functionally active human Mut-S-Homologon-2 in malignancies of the uterine corpus may enhance DNA repair capacity and increase the resistance of tumour cells to radiotherapy or chemotherapy. Upregulation of functionally active human Mut-S-Homologon-2 may be a mechanism at least in part responsible for the lack of effectiveness of various chemotherapy regimens in sporadic endometrial carcinomas. The analysis of tumours for DNA repair capacity including human Mut-S-Homologon-2 status might be of value in predicting the therapeutic response to chemotherapy or radiation. Both the treatment and the prognosis of endometrial carcinoma are strongly related to the stage of the disease (5-year survival rate for stage I endometrial carcinoma: 82-95%; for stage II endometrial carcinoma: 50-60%; for stage III endometrial carcinoma: 15-25%; for stage IV endometrial carcinoma: <10%) [40, 41]. Standard treatment for patients with early-stage disease is surgery with or without adjuvant radiation therapy [42]. It is still debatable, whether all patients should undergo pelvic or paraaortic lymph node dissection or whether this treatment should be reserved for patients with high risk factors [43].

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